Significance of ZO-1, an Intercellular Adhesion Molecule, as a Prognostic Marker in Lung Adenocarcinoma

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In epithelial tissues, intercellular adhesion structures are formed between adjacent cells *via* intercellular adhesion factors, such as zonula occludens (ZO-1), to maintain the structure and function of tissues and organs, thereby contributing to homeostasis. Epithelial cells are polarized into apical and basal regions by tight junctions (TJs), a type of intercellular adhesion structure, and thus, their intracellular organelles are asymmetrically distributed. Normal epithelial cells maintain their cellular function by controlling cytoskeletal reorganization, motility, and division by maintaining asymmetry in their intracellular organelles. Among the features common to many cancer tissues are abnormalities in cell polarity and intercellular adhesion. Lung adenocarcinoma consists of a mixture of five different histologic types that can be distinguished in the same section: lepidic, papillary, acinar, micropapillary, and solid patterns. Therefore, it is often difficult to accurately assess histological images because the staining differs according to the histological types. In the present study, we evaluated ZO-1 staining based on histological features observed in a single section and examined its relationship to clinicopathological features. In non-tumor areas, ZO-1 was expressed on the plasma membrane and in the cytoplasm of normal alveolar epithelial cells. However, in tumor areas, ZO-1 staining was mainly localized in the cytoplasm and on the plasma membrane only in a few cells. ZO-1-negative cases tended to have poorer prognoses in all histological types, with a poorer prognosis in the solid pattern.

These results suggest that ZO-1 expression in solid-pattern lung adenocarcinoma may be a useful prognostic marker.

Key words: Lung adenocarcinoma, intercellular adhesion, tight junctions, immunohistochemistry, prognostic marker

INTRODUCTION

In Japan, lung cancer is the most common cause of death among malignant tumors, and more than half of the patients have stage III or more advanced cancer at the time of diagnosis. Even in early-stage cases that have been treated with surgery or adjuvant chemoradiotherapy, many patients develop recurrent or metastatic disease and have a poor prognosis. In recent years, molecular targeted drugs, such as bevacizumab, erlotinib, and gefitinib, and immune checkpoint inhibitors, such as nivolumab, pembrolizumab, and atezolizumab, have been developed for lung adenocarcinoma, which accounts for approximately half of all lung cancers. Their survival outcomes have improved, but the prognosis is still worse than for other cancers in other organs [1]. Understanding the molecular mechanisms of pathogenesis and progression of lung adenocarcinoma will contribute to the development of molecular diagnostic markers and the identification of therapeutic targets. It will also play an important role in the selection of appropriate treatment based on the estimated prognosis and in the evaluation of sensitivity to anticancer drugs.

Lung cancer is classified into two major types based on histological characteristics and treatment options: small cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). NSCLC is further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The WHO classification (5th edition) published in 2015 changed the classification of lung adenocarcinoma [2]. The first change was the deletion of the term bronchioalveolar epithelial cancer and the introduction of the classification of tumors of 3 cm or less with pure lepidic growth as intraepithelial adenocarcinoma (AIS) and tumors of 3 cm or less with predominantly lepidic growth and an infiltrated area of 5 mm or less as microinvasive adenocarcinoma (MIA). In addition, the term "mixed adenocarcinoma" was abolished and invasive adenocarcinoma was subclassified into five subtypes according to the predominant growth histologic pattern. Prognosis is found to be poor in the following order: low-grade AIS / MIA group < intermediate-grade alveolar replacement / papillary pattern / lepidic pattern group < high-grade micropapillary pattern / solid pattern group; the new

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classification is consistent with the prognosis.

The cells that comprise multicellular organisms are broadly classified into epithelial and mesenchymal cells. Epithelial cells adhere to each other *via* cell adhesion molecules with different functions and structures, such as tight junctions (TJs), adherens junctions, gap junctions, and desmosomes between adjacent cells [3]. Epithelial cells are polarized, with two regions having different functions, apical and basolateral, bordering the TJs, and they maintain tissue structure by being oriented differently [4]. TJs have two major functions: a fence function that zones and maintains the cell membrane so that the components of membrane proteins and lipid components specific to each region do not intermingle and a gating function that regulates the passage of substances between cells [5]. The ZO (zonula occludens) family proteins (ZO-1,2,3), which are components of TJs, play an important role in the formation of intercellular adhesion and cell polarity [6]. ZO-1 is a major molecule of the ZO family proteins expressed in most epithelial tissues [7]. ZO-2 and 3 are expressed in certain epithelial tissues, such as the oral epithelium, female genitalia, and the urinary system [7]. ZO-1 is involved not only in the maintenance of cell polarity but also in the regulation of gene transcription, cell proliferation, and tumor cell metastasis [8]. In hepatocellular carcinoma tissues, the suppression of ZO-1 expression is associated with increased cell proliferation and metastasis [9]. Malignant tumors often show a disruption of cell polarity and intercellular adhesion, which leads to the loss of apical-basal polarity, as well as to tumor cell invasion and metastasis [10]. These findings suggest that decreased expression of ZO-1 is closely related to tumor cell proliferation and invasive metastasis [9].

Although ZO-1 expression is associated with NSCLC or squamous cell carcinoma of the lung [11, 12], the prognostic impact of ZO-1 expression in lung adenocarcinoma is unknown (Fig. 3A). As each histological pattern on the same section often shows different levels of staining, it is difficult to accurately evaluate the different patterns of lung adenocarcinoma during immunohistochemical examinations. Therefore, in the present study, we evaluated ZO-1 staining based on the histology of different patterns on the same section of lung adenocarcinoma and then, examined its association with prognosis to determine its potential as a marker for prognostic estimation.

MATERIALS AND METHODS

Clinicopathological data

A total of 174 patients with adenocarcinoma of the lung who underwent radical surgery for lung cancer at Tokai University Hospital between 2000 and 2005 were included in the study. After receiving informed consent from the patients, tissue specimens were prepared from surgically resected adenocarcinomas and analyzed. The subclassification of histologic types of invasive adenocarcinoma was performed according to the WHO Classification (6th edition). The median postoperative observation period was 2,069 (12 to 6,592) days. This study was conducted with the approval of the Institutional Ethics Committee of Tokai University Hospital (IRB No. 11R-002).

Immunohistochemistry

Paraffin-embedded sections of 4-μm thickness were prepared from the tissue specimen. The paraffin was removed using xylene and ethanol and immersed in 0.01 M phosphate-buffered saline (PBS). The sections used for immunostaining of ZO-1 were treated with protease (2 mg/ml, Type XIV from *Streptomyces griseus*, Sigma, USA) at room temperature for 10 minutes for antigenic retrieval followed by endogenous peroxidase blocking with 3% H_2O_2. Rabbit anti-human ZO-1 antibody (ThermoFisher Scientific, MA, USA) was diluted (1:50) with Tris-buffered saline containing 10% Tween-20 and the sections were incubated at 4°C overnight. Next, the sections were incubated with Simple Stain anti-rabbit antibody (Nichirei Bioscience, Japan) at room temperature for 30 minutes. After washing with PBS, 3'3-diaminobenzidine tetrahydrochloride (DAB) was added to facilitate color development.

Evaluation of immunostaining

For each case of lung adenocarcinoma, the intensity of ZO-1 immunostaining was determined for each histological image to identify lepidic, papillary, acinar, micropapillary, and solid patterns. Positive results were defined as sections with staining of 10% or more of the area, while negative results were defined as those with staining of less than 10% of the area.

Statistical analysis

Univariate analysis (χ-square test, Student's *t*-test) was considered significant at $P < 0.05$. The survival period was defined as the number of days from the date of surgery to the date of death, including all causes (without any distinction between lung cancer and death from other diseases). The relationship between ZO-1 expression and the survival rate was evaluated using Kaplan-Meier survival curves. The log-rank test was used for the survival curves. All analyses were performed using IBM SPSS Statics (version 26; IBM Japan, Japan).

RESULTS

Clinicopathologic features of the cases

The clinicopathologic features are shown in Table 1. The subjects were 174 patients who underwent surgery (lobectomy and mediastinal lymph node dissection) for lung adenocarcinoma between 2000 and 2005 at Tokai University Hospital. Of these 174 patients, 84 were male and 90 female (23 to 77 years; median age 64 years). The maximum tumor diameter ranged from 4 to 75 mm, with a median diameter of 24 mm. Of the 174 cases, 87 were in stage IA, 23 in stage IB, 6 in stage IIA, 9 in stage IIB, 29 in stage IIIA, 17 in stage IIIB, and 3 in stage IV (UICC pathological TNM (pTNM) classification (version 6)).

Association between ZO-1 staining and clinicopathological features for each pattern of lung adenocarcinoma

In non-tumor areas, ZO-1 was found to be expressed in the plasma membrane and cytoplasm of normal alveolar epithelial cells (Fig. 1A). However, in tumor areas, staining was mainly localized in the cytoplasm, with only a few cells showing staining on the plasma membrane (Fig. 1B). Fig. 2 shows negative

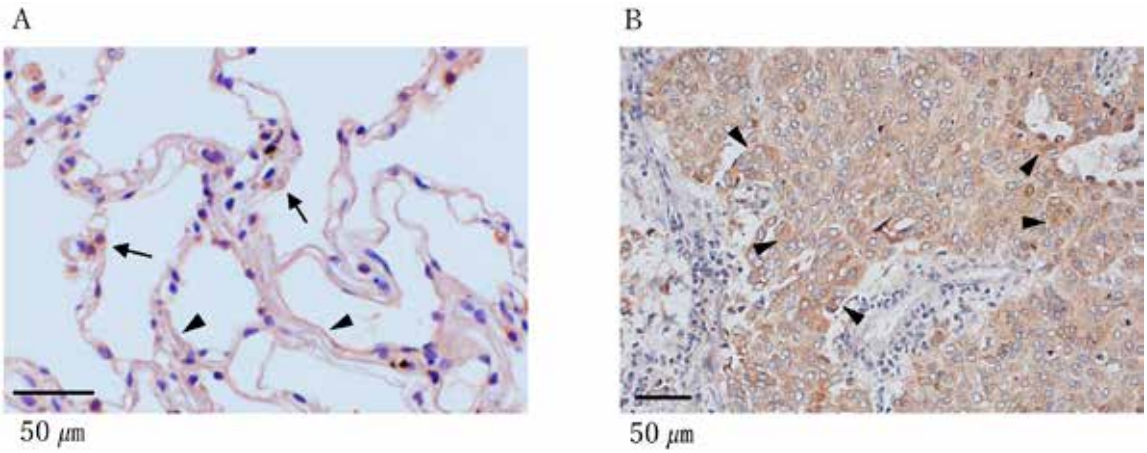

Fig. 1 Immunohistochemical images of normal and tumor areas in human lung tissue. (Magnification: 100×)
(A) ZO-1 staining images of normal areas: arrows indicate a stained area in the cytoplasm and arrowheads indicate a stained area in the plasma membrane.
(B) ZO-1 staining image of tumor site: arrows indicate an area stained with cytoplasm.

and positive cases of immunostaining for ZO-1 for each pattern in each histological image. ZO-1 was found mainly in the cytoplasm in each histological image. The results of ZO-1 immunostaining were evaluated for each histological image (lepidic, papillary, acinar, micropapillary, and solid patterns). Fig. 3 shows Kaplan-Meier survival curves (overall survival) for the ZO-1-positive and -negative groups for all cases (Fig. 3A) and each histological image (Fig. 3B–F). There was no significant difference between ZO-1 -positive and -negative groups for all cases. However, the ZO-1-negative group with solid pattern histology had a significantly shorter survival time than the ZO-1-positive group ($p = 0.029$, Fig. 3F). Other histological findings also showed a trend toward shorter survival in the ZO-1-negative group than in the ZO-1-positive group (Fig. 3B–E). Table 8 shows that there was no significant difference in the 5-year relapse- free survival rate for the ZO-1-positive and -negative groups for each pathological stage.

Tables 2 to 6 show the correlation between ZO-1 expression for each histological image and each clinicopathological feature. Univariate analysis by χ-square analysis revealed that ZO-1-positive cases were significantly more common in males ($p = 0.049$) in the alveolar replacement pattern (Table 2). There was no association with other clinicopathological parameters (Tables 3 to 6). In multivariate analysis, there was no association with any of the clinicopathological features (Table 7).

DISCUSSION

The expression kinetics of cell polarity-related molecules such as aPKC, Par, and Hugl have a significant impact on invasion, metastasis, and survival outcome of NSCLC, including lung adenocarcinoma [12–14]. However, the association between the prognosis of lung adenocarcinoma and the expression of intercellular adhesion molecules is unclear. In addition, lung adenocarcinomas exhibit a mixture of different histological features (lepidic, papillary, acinar, micropapillary, and solid patterns) in the same section, hindering an accurate immunohistochemical analysis. In this study, we evaluated the expression of ZO-1 on the same sections for each histological pattern and correlated it with the clinicopathological features. Thus, we report, for the first time, that the prognosis was worse in the ZO-1-negative group in the solid pattern tissue sites than in the ZO-1-positive group. Thus, the level of ZO-1 expression at the sites of the solid pattern could be considered a very useful marker for estimating the prognosis of lung adenocarcinoma.

Several reports have described the association between ZO-1 expression and prognosis in various cancers [11, 15–17]. ZO-1 expression is reduced by 69% in breast cancer patients compared to normal patients and even by 93% in poorly differentiated patients [15]. In gastrointestinal cancers, ZO-1 expression has also been found to be decreased in adenocarcinomas of the stomach and colon compared to normal tissues, the decrease being proportional to the lack of differentiation [16]. Furthermore, ZO-1 expression is decreased in liver metastases of colorectal cancer [17]. Conversely, the increased expression of ZO-1 is associated with a better prognosis in NSCLC, including lung adenocarcinoma and squamous cell carcinoma of the lung [11]. The results of this study showed that a decreased expression of ZO-1 in solid pattern tissue sites is a poor prognostic factor, and the evaluation of staining for each histological pattern on the same section in lung adenocarcinoma will be an important point when considering the selection of other candidate prognostic markers. Of note, tissues showing a micropapillary pattern, a histological type of a poorly differentiated lung adenocarcinoma, also showed a trend toward worse prognosis with decreasing ZO-1 expression.

In normal tissues, ZO-1 is located in TJs in the plasma membrane. Our results revealed that ZO-1 is expressed in the cytoplasm of lung adenocarcinoma tissue but not in the plasma membrane. Cell polarity cannot be maintained when cells become cancerous, suggesting that ZO-1 has lost its localization at the plasma membrane in lung adenocarcinomas. The cytoplasmic expression of ZO-1, which is normally located in TJs, may result in weakened intercellular adhesion and failure to maintain cell polarity, leading to in-

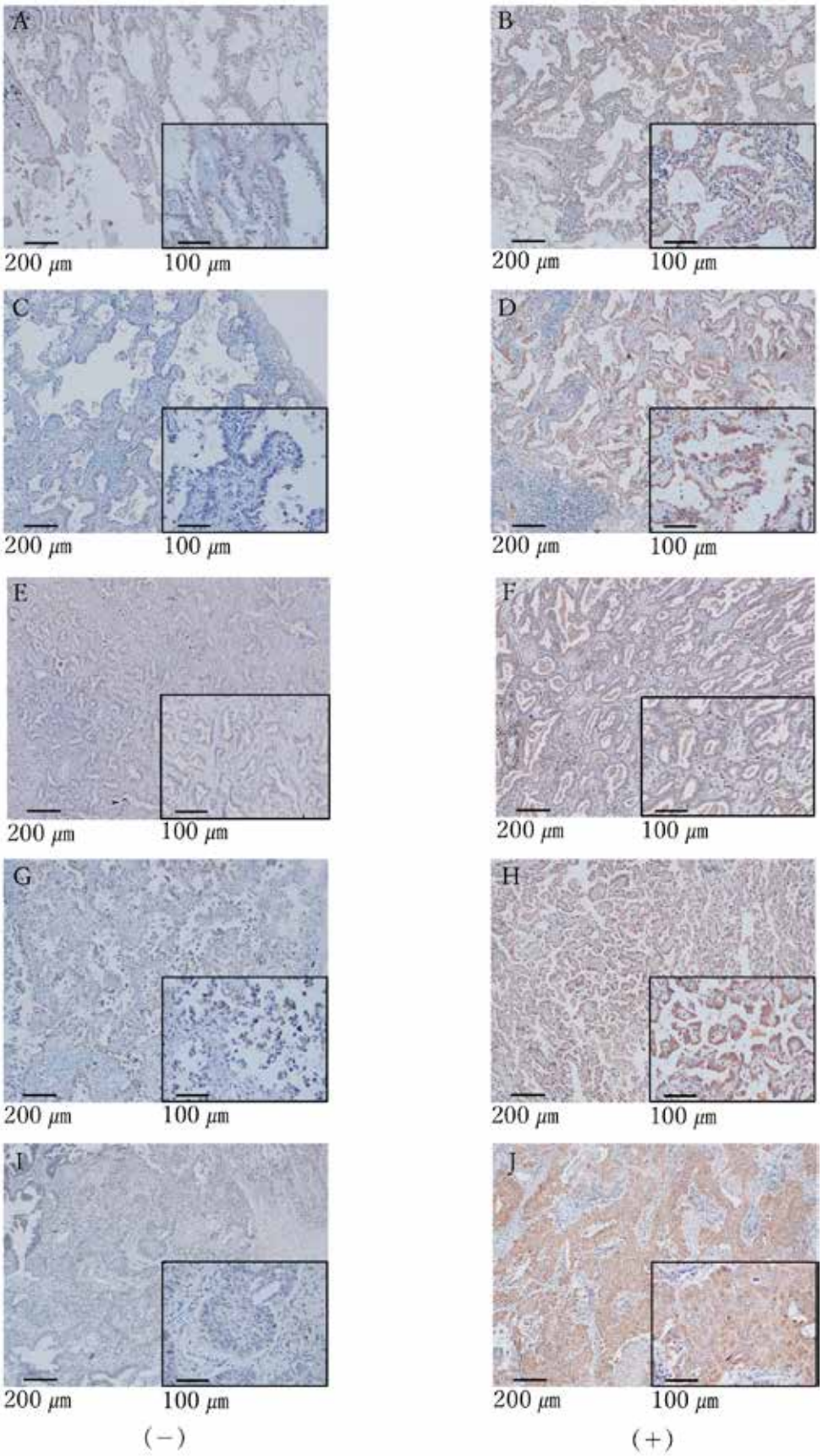

Fig. 2 Immunohistochemical images of ZO-1 at tumor lesions in human lung tissue (magnification: 40×). The lower right photo is a strongly magnified view.

(A) ZO-1-negative images in lepidic pattern
(B) ZO-1-positive images in lepidic pattern
(C) ZO-1-negative images in papillary pattern
(D) ZO-1-positive images in papillary pattern
(E) ZO-1-negative images in acinar pattern
(F) ZO-1-positive images in acinar pattern
(G) ZO-1-negative images in micropapillary pattern
(H) ZO-1-positive images in micropapillary pattern
(I) ZO-1-negative images in solid pattern
(J) ZO-1-positive images in solid pattern

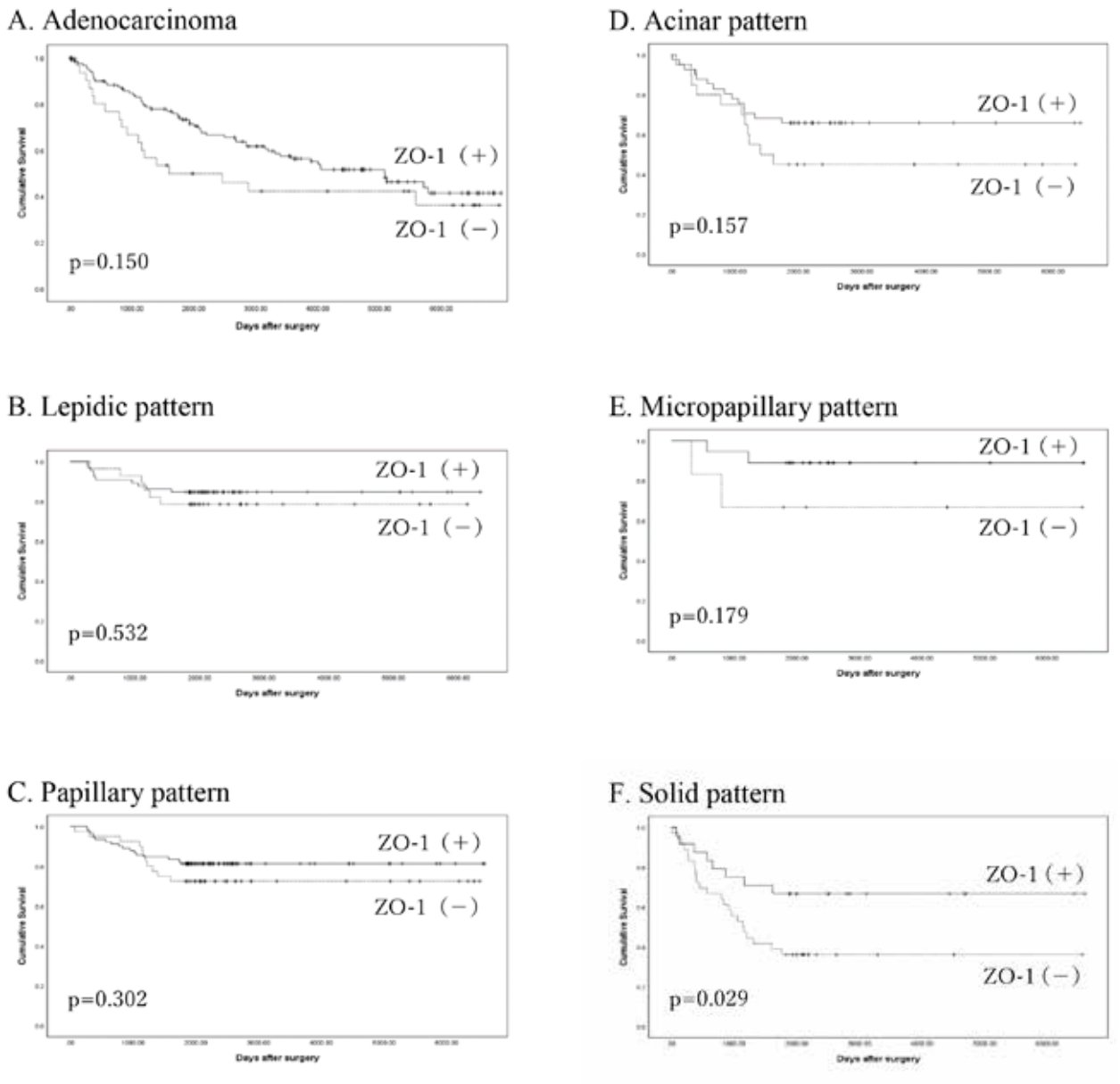

Fig. 3 Kaplan-Meier survival curves for ZO-1 staining by histological image.
(A) Comparison of ZO-1-positive and -negative groups in adenocarcinoma
(B) Comparison of ZO-1-positive and -negative groups in lepidic pattern
(C) Comparison of ZO-1-positive and -negative groups in papillary pattern
(D) Comparison of ZO-1-positive and -negative groups in acinar pattern
(E) Comparison of ZO-1-positive and -negative groups in micropapillary pattern
(F) Comparison of ZO-1-positive and -negative groups in solid pattern

creased cancer cell invasiveness and a poor prognosis.

Immunohistochemical studies have shown that ZO-1 is primarily localized to the plasma membrane in squamous cell carcinomas of the lung. In addition, ZO-1 is clustered in parts of the cell membrane (foci), and the group showing these foci (the foci group) was found to have a significantly larger tumor size and, higher recurrence, and mortality rates than the group without foci, indicating a poor prognosis [12]. However, unlike squamous cell carcinoma, ZO-1 was mainly localized in the cytoplasm in the adenocarcinoma examined in this study and was not clustered on the cell membrane. ZO-1 is one of the major molecules that form TJs and is present in adherens and gap junctions, thus playing an important role in intercellular adhesion. AMP-activated protein kinase (AMPK), aPKC, and Afadin contribute to the localization of ZO-1 at the plasma membrane. AMPK facilitates the binding of ZO-1 to Afadin via the phosphorylation of Afadin, and the ZO-1/Afadin complex is transferred to the adherens junction following Afadin phosphorylation by aPKC [18, 19]. Afadin then dissociates from ZO-1, and ZO-1 translocates to a TJ. Thus, differences in the expression and function of AMPK, aPKC, and Afadin, which are involved in the translocation of ZO-1 to the plasma membrane, can result in differences in the intracellular expression of ZO-1 between lung adenocarcinoma and lung squamous cell carcinoma. Understanding the

Table 1 Clinicopathologic background of lung adenocarcinoma cases

Age (years)		64 (23-77)
Gender	Male/Female	84/90
Tumor size (mm)		25 (4-75)
Tumor status	T1/T2/T3/T4	110/46/3/15
Nodal metastasis	N0/N1/N2/N3	121/17/34/2
Metastasis	M0/M1	171/3
Stage	1A/1B/2A/2B/3A/3B/4	87/23/6/9/29/17/3
Observation time (day)		2069 (12-6592)

Table 2 Correlation of ZO-1 staining of lepidic patterns with clinicopathologic features

	n (%)	Negative	Positive	χ^2
Age at surgery (years)				
< 64	52 (52.5)	17	35	0.586
≧64	47 (47.5)	13	34	
Gender				
Male	41 (41.4)	8	33	0.049
Female	58 (58.6)	22	36	
Tumor size				
≦30	75 (75.8)	22	53	0.711
> 30	24 (24.2)	8	16	
Lymph node metastasis				
Negative	76 (76.8)	23	53	0.987
Positive	23 (23.2)	7	16	
Metastasis				
Negative	97 (98.0)	29	68	0.516
Positive	2 (2.0)	1	1	

Table 3 Correlation of ZO-1 staining of papillary pattern with clinicopathologic features

	n (%)	Negative	Positive	χ^2
Age at surgery (years)				
< 64	72 (52.2)	21	51	0.598
≧64	66 (47.8)	22	44	
Gender				
Male	60 (43.5)	17	43	0.53
Female	78 (56.5)	26	52	
Tumor size				
≦30	98 (71.0)	31	67	0.851
> 30	40 (29.0)	12	28	
Lymph node metastasis				
Negative	100 (72.5)	30	70	0.633
Positive	38 (27.5)	13	25	
Metastasis				
Negative	136 (98.6)	42	94	0.528
Positive	2 (1.4)	1	1	

mechanism of the subcellular localization of ZO-1, a molecule essential for the formation and maintenance of intercellular adhesion and cell polarity, will be an important topic for further investigation.

ZO-1 localizes to TJs and plays an important role in intercellular adhesion, whereas in lung adenocarcinomas, it localizes to the cytoplasm, suggesting a dysfunction of this molecule. Furthermore, lung adenocarcinomas with a solid pattern, a type of poorly differentiated histology, have decreased ZO-1 expression, which is presumably more conducive to tumor invasion and a poorer prognosis.

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Table 4 Correlation of ZO-1 staining of acinar pattern with clinicopathologic features

	n (%)	Negative	Positive	χ^2
Age at surgery (years)				
< 64	29 (45.3)	11	18	0.866
≧64	35 (54.7)	14	21	
Gender				
Male	35 (54.7)	15	20	0.494
Female	29 (45.3)	10	19	
Tumor size				
≦30	39 (60.9)	14	25	0.517
> 30	25 (39.1)	11	14	
Lymph node metastasis				
Negative	39 (60.9)	14	25	0.517
Positive	25 (39.1)	11	14	
Metastasis				
Negative	63 (98.4)	24	39	0.391
Positive	1 (1.6)	1	0	

Table 5 Correlation of ZO-1 staining of micropapillary patterns with clinicopathologic features

	n (%)	Negative	Positive	χ^2
Age at surgery (years)				
< 64	17 (60.7)	5	12	1.000
≧64	11 (39.3)	3	8	
Gender				
Male	12 (42.9)	2	10	0.401
Female	16 (57.1)	6	10	
Tumor size				
≦30	22 (78.6)	5	17	0.311
> 30	6 (21.4)	3	3	
Lymph node metastasis				
Negative	19 (67.9)	4	15	0.371
Positive	9 (32.1)	4	5	
Metastasis				
Negative	28 (100.0)	8	20	N/A
Positive	0 (0.0)	0	0	

Table 6 Correlation of ZO-1 staining of solid patterns with clinicopathologic features

	n (%)	Negative	Positive	χ^2
Age at surgery (years)				
< 64	28 (44.4)	18	10	0.306
≧64	35 (55.6)	18	17	
Gender				
Male	41 (65.1)	22	19	0.446
Female	22 (34.9)	14	8	
Tumor size				
≦30	35 (55.6)	17	18	0.124
> 30	28 (44.4)	19	9	
Lymph node metastasis				
Negative	32 (50.8)	16	16	0.244
Positive	31 (49.2)	20	11	
Metastasis				
Negative	61 (96.8)	34	27	0.502
Positive	2 (3.2)	2	0	

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Table 7 Multivariate analysis of prognostic factors in adenocarcinoma with solid patterns

variable	p-value	Hazard ratio	95% confidence interval
ZO-1 expression Positive vs Negative	0.083	1.866	0.922-3.780
Gender Male vs Female	0.145	1.734	0.826-3.639
Age (years) ＜64 vs ≧64	0.222	0.642	0.315-1.308
Tumor size (mm) ＜30 vs ≧30	0.111	0.489	0.244-0.981
Lymph node metastasis Positive vs Negative	0.076	0.447	0.184-1.087
M status Positive vs Negative	0.056	0.192	0.035-1.043
pStage Ⅰ-Ⅱ vs Ⅲ-Ⅳ	0.285	0.786	0.506-1.222

Table 8 Five-year relapse free survival rate (5-RFS) for the ZO-1-positive and -negative groups for each pathological stage

Stage	ZO-1(+)	ZO-1(-)	p-value
ⅠA	61/72 (84.7%)	14/15 (93.3%)	0.498
ⅠB	12/16 (75.0%)	4/7 (57.1%)	0.310
ⅡA	1/5 (20.0%)	1/1 (100%)	0.259
ⅡB	0/6 (0.0%)	1/3 (33.3%)	0.500
ⅢA	4/22 (18.2%)	2/7 (28.6%)	0.422
ⅢB	3/11 (27.3%)	2/6 (33.3%)	0.435

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